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(54) Title: GENETIC METHOD FOR PRODUCING VIRUS RESISTANT ORGANISMS

(57) Abstract

The present invention relates to a method of producing a host organism which is resistant to infection by a virus comprising stably incorporating into the genome of said host a DNA sequence encoding a non–viral protein characterized in that said protein has the ability to bind to ssRNA from said virus to form a non–translatable RNA–protein complex. The host organism is preferably a plant. The invention further relates to transgenic plants and plant cells having stably incorporated into its genome a DNA sequence encoding a non–viral protein having the ability to bind to ssRNA from said virus to form a non–translatable RNA–protein complex and to DNA sequences and constructs for use in the method.

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GENETIC METHOD FOR PRODUCING VIRUS RESISTANT ORGANISMS

This invention relates to a method of producing virus resistant organisms, in particular plants, to cells transformed with DNA coding for single strand RNA binding proteins, to DNA coding for said single strand RNA binding proteins and to vectors containing said DNA.

During the past decade evidence has accumulated that plant virus-encoded movement proteins (MPs) are required to mediate viral spread between plant cells via plasmodesmata (PD) (reviewed by Lucas and Gilbertson, Ann. Rev. Phytopathol. 32, 397-411 (1994)). Different viruses use distinct strategies for coding their MPs (reviewed by Atabekov and Taliansky, Advan. Virus Research 38, 201-248 (1990)). In particular, the movement of tobamoviruses is mediated by the 30K protein encoded by a single gene (Deom et al., Proc. Natl. Acad. Sci. USA 87, 3284-3288 (1990); Meshi et al., EMBO J. 6, 2557-2563 (1987)), whereas that of potexviruses, hordeiviruses, carlaviruses and some furo-like viruses requires the products of the three overlapping genes (triple gene block, TGB)(for review, see Donald et al., In Fifth International Symposium on Biotechnology and Plant Protection: Viral Pathogenesis and Disease Resistance pp 135-147, 1994). The 5'-proximal TGB ORF codes for the protein possessing the nucleotide binding domain, and the second and third TGB ORFs encode small hydrophobic proteins (Morozov et al., Biochimie 72, 677-84, (1990); Jackson et al., Seminars in Virology 2 107-119 (1991)).

Several viral MPs have been localized to PD in infected plants as well as in transgenic plants that express MP genes (Tomenius et al., Virology 160, 363-71 (1987); Berna et al., Virology 182, 682-89 (1991); Ding et al., Plant Cell 4 915-28 (1992); Fujiwara et al., Plant Cell 5, 1783-94 (1993)), i.e. they need to possess a PD localization signal to interact with hypothetical host factors (HF) (presumably, the structural proteins of PD). Two more activities of MPs have been demonstrated:

(i) their ability to increase plasmodesmatal size exclusion limit (SEL) (Wolf et al., Science 246, 377-79 (1989); Plant Cell 3, 593-604, (1991); Fujiwara et al., Plant Cell 5, 1783-94 (1993); Noueiry et al., Cell 76, 925-32 (1994); Waigmann et al., Proc. Natl. Acad. Sci. USA 91, 1933-1937 (1994), Ding et al., Virology 207, 345-53 (1995); Waigmann and Zambrysky, Plant Cell 7, 2069-2079 (1995); Angell et al., Virology 216 197-201 (1996)) and (ii) their sequence-independent binding of single-stranded nucleic acids (Citovsky et al., Cell 60, 637-647, (1990); Plant Cell 4, 397-411 (1992); Osman et al., J. Gen. Virol. 74, 2453-57, (1993); Schoumacher et al., Virology 188 896-99 (1992); J. Gen. Virol. 75, 3199-3201 (1994); Giesman-Cookmeyer and Lommel, Plant Cell 5, 973-982, (1993); Li and Palukaitis, Phytopathology 83, 1425 (1993); Virology 216, 71-79 (1996); Rouleau et al., Virology 204, 254-65 (1994); Pascal et al., Plant Cell 6, 995-1006 (1994); Offei et al., J. Gen. Virol. 76, 1493-96 (1995); Bleykasten et al., J. Gen. Virol. 889-897 (1996)). The most thoroughly studied virus-encoded MP is that of common tobamovirus (TMV UI) (Citovsky et al., 1990; 1992 J. loc. cit.). A new tobamovirus systemically infecting crycifer plants (cr TMV) has been recently isolated and the crTMV genome has been sequenced (Dorokhov et al., FEBS Lett. 350, 5-8 1994). It was reported that binding of the recombinant crTMV 30K MP to genomic viral RNA results in the formation of stable ribonucleoprotein complexes <u>in vitro</u> (Ivanov et al., FEBS Lett. 346, 217-220 1994). The TGB-coded 58K MP of barley stripe mosaic hordeivirus (BSMV) has co-operative RNA-binding activity <u>in vitro</u> (Donald et al., 1994 In Fifth International Symposium on Biotechnology and Plant Protection: Viral Pathogenesis and Disease Resistance pp 135-47) and forms a RNP-complex with viral RNA <u>in vivo</u> (Brakke et al., J. Gen. Virol. 69, 481-89, (1988)). The TGB-coded counterpart of BSMV 58K MP in potato virus X (PVX) genome is the 25K MP (Donald et al., 1994 J. loc cit.). The RNA-binding activity of the 25K MP is

negligible and can be detected only at very low salt concentrations (Kalinina et al., unpublished data). Thus, the MPs of tobamovirus and the largest TGB-coded MPs of hordei- and potexviruses are structurally (and presumably, functionally) distinct proteins with apparently varying RNA-binding activities.

It has been proposed by Citovsky and Zambrysky (1991) (Bioessays 13, 373-79) that viral MP and genomic RNA form an extended, linear ribonucleoprotein (RNP) complex which should be targeted to and translocated through plasmodesmata (PD). Further, it has been speculated that the MP-RNA complexes represent a particular pool of viral RNA molecules which are excluded from replication being designed for translocation (Citovsky et al., Cell 60, 637-47 (1990)). It is likely that the MP- and viral RNA-containing RNP represents a complex that binds to putative plasmodesmal receptors (HFs).

It is reasonable to hypothesize that once the viral RNA has been associated with the MP molecules into RNP, it becomes nontranslatable, i.e. that the TMV 30K MP is able to function as a translational repressor. The present inventors have found that the MPs of the tobamoviruses were able to block translation of viral RNAs in vitro. It was proposed that such MP-RNA complexes: (i) are not infectious in the primary infected cells and (ii) can be converted into the translatable and replicatable form in the course of translocation through the plasmodesmata (Dorokhov et al., (1996) Dolkady Rossiyskoy Akademii Nauk 349, 259-61).

It was now found that some non-viral single-strand RNA binding proteins have the ability to bind non-specifically to viral RNA and to inhibit its in vitro translation. Due to the lack of signals for plasmodesmatal receptor recognition the RNP complex pre-formed in vitro is non-translatable and non-infective in planta. Similarly in animal cells ssRNA binding proteins

may bind to viral mRNAs preventing their translation and thereby blocking the early steps of virus replication.

Accordingly in a first aspect the invention provides a method of producing a host organism which is resistant to infection by a virus comprising stably incorporating into the genome of said host a DNA sequence encoding a non-viral protein characterized in that said protein has the ability to bind to ssRNA from said virus to form a non-translatable RNA-protein complex.

In a preferred embodiment of the first aspect of the invention there is provided a method of producing plants which are resistant to infection by a virus comprising stably incorporating into the genome of said plant a DNA sequence encoding a non-viral protein characterized in that said protein has the ability to bind to ssRNA from said virus to form a non-translatable RNA-protein complex.

Examples of suitable non-viral proteins include the SSB protein which is able to bind ssRNA and DNA non-specifically (Citovsky et al (1990) Cell 60, 637-47) and has no nuclear localization signals; the 56-60 kDa proteins which are responsible for global repression of mRNA in Xenopus oocytes and rabbit reticulocytes and have been identified as belonging to the Y box family of transcription factors (Murray et al., Proc. Natl. Acad. Sci. USA 89, 11-15 (1992); Deschamps et al., J. Biol. Chem. 267, 13799-13802 (1992); Tafuri and Wolffe, J. Biol. Chem. 268, 24255-24261 (1993); Edvokimova et al., J. Biol. Chem. 270, 3186-92 (1995)) and the reticulocyte 50kDa protein (p50) (Minich et al., Eur. J. Biochem. 212, 633-38 (1993)). The latter are for example found in mammalian reticulocytes from rabbit and rat. The molecular weight of p50 from rabbit calculated from its gene sequence is 38 kDa (Evdokimova et al., J. Biol. Chem. 270, 3186-3192 (1995)). The M_r of natural p50 isolated from reticulocytes is found to be 50 kDa on SDS-PAGE apparently due to in vivo modification(s) of p50 and/or an abnormal electrophoretic mobility. Translation

in rabbit reticulocyte lysate of in vitro RNA transcript of p50 gene gave rise to a major band of M_r of about 38 kDa.

The ssRNA binding proteins for use in the method of the invention may bind specifically to the viral mRNA or may be able to bind to multiple RNA types.

The non-translatability of the RNA-protein complex in plants may be due to the lack of disassociation or destabilization of the RNA-protein complex on passage through the plasmodesmata caused for example by the inability of the non-viral protein to bind to receptors present in the plasmodesmata. The non-viral protein may be inherently unable to bind to plasmodesmatal receptors or may have been altered genetically or chemically such that it can no longer functionally interact with the receptors.

It is desirable that the ssRNA binding protein is present in the transgenic host in excess relative to the viral mRNA in order to achieve optimal translation inhibition. The molar excess required will vary depending on the ssRNA binding protein used. The molar excess required will generally be in the range from 2-fold to 250-fold molar excess. The excess required may be quantified directly in vitro (in cell free protein synthesizing systems) in for example protoplast inoculation and plant inoculation experiments using RNP complexes formed at different protein:RNA ratios.

The invention further provides a DNA sequence encoding a non-viral protein for use in the method of the invention wherein said protein has the ability to bind ssRNA from a virus to form a non-translatable RNA-protein complex. The DNA sequence may be predicted from the known amino acid sequence and DNA encoding the protein may be manufactured using a standard nucleic acid synthesizer. The DNA sequence may be incorporated into a DNA construct or vector in combination with suitable regulatory sequences (promoter, terminator, enhancer, nuclear localization signal etc.). The DNA sequence may be placed

under the control of a homologous or heterologous promoter which may be a constitutive or an inducible promoter (stimulated by, for xample, environmental conditions, presence of a pathogen, presence of a chemical). The promoter can be a promoter for pathogenesis-related proteins where induction of the promoter should occur at the very early stages of virus replication in order to prevent general toxicity of the P50 gene product when expressed from a constitutive or developmentally regulated promoter.

Therefore, the expression of for example the P50 gene can be made specific for cells in which a viral infection cycle takes place. This can be achieved by making use of viral subgenomic promoters which are promoters that can only be recognized as being a site for transcription initiation by a viral replicase. As the interaction of the replicase and the subgenomic promoter is virus-specific, a subgenomic promoter can be derived from the virus for which resistance is desired.

Viral subgenomic promoters are genetic elements which function as cis-acting sequences within the minus-strand copy of genomic viral RNA from which the synthesis of subgenomic messengers is initiated. An example of a viral subgenomic promoter shown to be active when linked to a non-viral gene is the one located in front of the coat protein gene of Potato Virus X (PVX). During the replication cycle, the viral replicase binds to the RNA strand of minus polarity to synthesize a functional coat protein messenger of plus polarity which can subsequently be translated into the coat protein. The open reading frame will then be expressed upon viral infection in cis when incorporated in the viral genome or in trans when expressed from a stably integrated nuclear gene. The basic principle of expressing foreign genes using a PVX based vector having the coat protein subgenomic promoter has been demonstrated by making use of the marker gene GUS (Chapman et al., (1992), The Plant Journal 2, 549-557). A subgenomic promoter will only be active when the

replication takes place. When the virus is present as such (without being active) the promoter is silent.

In the case of DNA viruses a nuclear localisation signal may be present. The nuclear localization signal may be homologous or heterologous to the non-viral protein and will be chosen to ensure entry of the non-viral single stranded RNA binding protein into the nucleus to facilitate formation of non-translatable complexes with mRNAs derived from the infecting DNA-containing viruses before their export to the cytoplasm. The use of nuclear localization signals is especially preferred.

Such a DNA construct may be cloned or transformed into a biological system which allows expression of the encoded protein or an active part thereof. Suitable biological systems include yeast; cultured cells (such as insect cells, mammalian and plant cells) and plants and animals. In some cases, the expressed protein may subsequently be extracted and isolated for use.

For practical applications the protein may be used to improve the viral resistance of animals and may be used in agriculture to protect crops during the life of the plant. The protein may protect the transgenic organisms from future viral attack.

Plant cells may be transformed with recombinant DNA constructs according to a variety of known methods (Agrobacterium Ti plasmids, PEG mediated protoplast transformation, electroporation, microinjection, microprojectile gun, whiskers). The transformed cells may then in suitable cases be regenerated into whole plants in which the new nuclear material is stably incorporated into the genome and said transgenic plants form a further feature of the invention. Both transformed monocotyledonous and dicotyledonous plants may be obtained in this way.

The invention further provides a host organism having improved resistance to viral infection said host

containing recombinant DNA which expresses a non-viral protein capable of binding to ssRNA from said virus to form a non translatable RNA-protein complex. The host organism is preferably a plant cell.

A transgenic plant of the invention may be used as a parent in standard plant breeding crosses to develop hybrids and lines having improved viral resistance.

The invention extends also to seeds and progeny derived from the transgenic plants according to the invention wherein said seeds and progeny show improved resistance to viral infection and have stably incorporated into their genome a recombinant DNA sequence encoding a non-viral protein characterized in that said protein has the ability to bind to ssRNA from said virus to form a non-translatable RNA-protein complex.

Examples of genetic modified plants which may be produced include field crops, cereals, fruit and vegetables such as canola, sunflower, tobacco, sugarbeet, grasses, cotton, soya, maize, wheat, barley, rice, sorghum, tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, melons, potatoes, carrot, lettuce, cabbage, onion.

The invention is further illustrated in the following non-limiting examples and with reference to the following figures in which:

Fig. 1: Translation inhibiting activity of the E.coli SSB protein in wheat germ extracts (WGE). TMV RNA and E.coli SSB protein at the molar ratio of 100:1 (lane 1), TMV RNA (75 μg/ml) (lane 2), no RNA added (lane 3). Molecular weight markers are shown on the right.

Fig. 2: Comparison of translation inhibiting activity of wt TMV, MP, deletion mutant DEL4 MP and the mammalian protein p50. TMV RNA and the protein were preincubated and then added to rabbit reticulocyte lysates (RRL): no RNA added (lane 1); DEL4 MP and RNA at the molar ratio of 500:1 (lane 2); p50 and RNA at the ratio of 100:1 (lane 3); DEL4 MP and RNA at the ratio of 100:1 (lane 4); wt TMV and RNA at the ratio of 100:1

(lane 5); TMV RNA (75 µg/ml) (lane 6). Molecular weight markers are shown on the right.

Fig. 3: Construction of rabbit p50 gene-containing Agrobacterium tumefaciens. GUS = ß-glucuronidase , 35S = 35S Cauliflower promoter, alpha/beta = PVX nontranslated 5'-leader sequence, T CaMV = Cauliflower Mosaic Virus polyadenylation signal.

EXAMPLES

EXAMPLE 1

Inhibition of cell-free translation by E. coli SSB in vitro

a) Cell Free Translation

In vitro translation in rabbit reticulocyte lysates (RRL) was as described by Pelham and Jackson (Eur. J. Biochem 67, 247-56 (1976)) with minor modifications. The translation mixture (25 µl final volume) contained 10 µl nuclease-treated lysate containing 1 mM $CaCl_2$ with hemin; 20 mM Hepes, pH 7.6; 1 mM ATP; 200 mM GTP; 2.5 mM magnesium acetate; 100 mM potassium acetate; 2 mM DTT; 15 mM creatine phosphate; 1 mg creatine phosphokinase; 5 mM cAMP; 2 mM EGTA; 3 µg yeast tRNA; 125 mM of each essential amino acid excluding methionine; 800 mCi/ml [^{35}S]-methionine (Amersham, .1000 Ci/µmol) and 40-100 mg/ml of virus RNA. Incubation was carried out at 30°C for 60 min. Translation in wheat germ extracts (WGE) was performed according to the manufacturer's (Promega) protocol in the presence of [^{35}S]-methionine for 60 min at 25°C. Radiolabeled translation products were analyzed by SDS-PAGE and localized by autoradiography on the dried gel.

b) Inhibition of cell-free translation

Viral RNA and E.coli SSB (Promega) were preincubated at different molar ratios in 15µl 0.01M Tris-HCl, pH 7.0 in the presence of human placenta RNase inhibitor on ice for 1h. Subsequently, 25 µl of RRL or WGE translation reaction containing [^{35}S]-methionine were

added and translation was allowed to proceed. Alternatively the SSB preparations and viral RNA were added without preincubation directly to the cell-free system.

c) In vitro transcription

Radiolabelled 1700-nt RNA fragment was prepared by in vitro transcription of the linearized plasmid that contained crTMV MP and CP genes and the 3'nontranslated region of crTMV RNA (Dorokhov et al., FEBS Lett. 350 5-8 (1994)). The plasmid was transcribed with T7 polymerase (Promega) in the presence of [α^{32}P]UTP as described in the manufacturer's protocol.

d) Nitrocellulose membrane filter binding (NFB) assays.

Double filter NFB assays were done as described by Wong and Lohman (1993) (Proc. Natl. Acad. Sci. USA 90, 5428-5432). About 300x10^3 cpm of the [^{32}P] transcript, 2 μg of unlabelled TMV RNA and different amounts of the E.coli SSB were mixed in 30 μl of binding buffer A (50 mM Tris-HCl, 1 mM EDTA, pH 7.0, 50 mM NaCl, 1 mM DTT, 1 mg/ml bovine serum albumin and 10% glycerol). After incubation on ice for 1h, the mixture was subjected to NFB assay. The mixture was filtered through two layers at 45-μm nitrocellulose membranes (upper layer of nitrocellulose blotting membrane Bio Trace NT and lower one of positively charged blotting membrane Bio Trace HP Gelman Sciences) using the Slot Blot device (Hoffer S.I.). The membranes were washed three times with 100 μl buffer A, dried and counted for radioactivity retained using a liquid scintillation counter (Beckman).

EXAMPLE 2

Inhibition of cell-free translation by reticulocyte p50 protein

a) Cell fre translation

In vitro translation in rabbit reticulocyte lysates (RRL) was as described by Pelham and Jackson

(Eur. J. Biochem. 67, 247-56 (1976)) with minor modifications. The translation mixture (25 µl final volume) contained 10 µl nuclease-treated lysate containing 1 mM $CaCl_2$ with hemin; 20 mM Hepes, pH 7.6; 1 mM ATP; 200 mM GTP; 2.5 mM magnesium acetate; 100 mM potassium acetate; 2 mM DTT; 15 mM creatine phosphate; 1 µg creatine phosphokinase; 5 mM cAMP; 2 mM EGTA; 3 µg yeast tRNA; 125 µg of each essential amino acid excluding methionine; 800 uCi/ml [^{35}S]-methionine (Amersham, .1000 Ci/µmol) and 40-100 µg/ml of virus RNA. Incubation was carried out at 30°C for 60 min. Translation in wheat germ extracts (WGE) was performed according to the manufacturer's (Promega) protocol in the presence of [^{35}S]-methionine for 60 min at 25°C. Radiolabeled translation products were analyzed by SDS-PAGE and localized by autoradiography on the dried gel.

b) Inhibition of cell-free translation

Viral RNA and reticulocyte 50 kDa protein (Minich et al., 1993 Eur. J. Biochem. 212 633-638) were preincubated at different molar ratios in 15µl 0.01M Tris-HCl, pH 7.0 in the presence of human placenta RNase inhibitor on ice for 1h. Subsequently, 25 µl of RRL or WGE translation reaction containing [^{35}S]-methionine were added and translation was allowed to proceed. Alternatively the 50 kDa preparations and viral RNA were added without preincubation directly to the cell-free system.

c) <u>In vitro</u> transcription

Radiolabelled 1700-nt RNA fragment was prepared by <u>in vitro</u> transcription of the linearized plasmid that contained crTMV MP and CP genes and the 3'nontranslated region of crTMV RNA (Dorokhov et al., FEBS Lett. 350 5-8 (1994)). The plasmid was transcribed with T7 polymerase (Promega) in the presence of [α^{32}P]UTP as described in the manufacturer's protocol.

d) Nitrocellulose membran filter binding (NFB) assays

Double filter NFB assays were done as described by Wong and Lohman (1993) (Proc. Natl. Acad. Sci USA 90 5428-32). About $300x10^3$ cpm of the [^{32}P] transcript, 2 µg of unlabelled TMV RNA and different amounts of the 50 kDa protein were mixed in 30 µl of binding buffer A (50 mM Tris-HCl, 1 mM EDTA, pH 7.0, 50 mM NaCl, 1 mM DTT, 1 mg/ml bovine serum albumin and 10% glycerol). After incubation on ice for 1h, the mixture was subjected to NFB assay. The mixture was filtered through two layers at 45-µm nitrocellulose membranes (upper layer of nitrocellulose blotting membrane Bio Trace NT and lower one of positively charged blotting membrane Bio Trace HP Gelman Sciences) using the Slot Blot device (Hoffer S.I.). The membranes were washed three times with 100 µl buffer A, dried and counted for radioactivity retained using a liquid scintillation counter (Beckman).

EXAMPLE 3

Inhibition of virus multiplication by reticulocyte 50 kDa protein in protoplasts and in planta

a) Plant inoculation

TMV RNA and the reticulocyte 50 kDa protein (Minich et al., Eur. J. Biochem. 212 633-638 (1993)) were preincubated at different molar ratios in 50 ml 0.01 M Tris-HCl, pH 7.5 in the presence of human placenta RNase inhibitor on ice for 1h. The opposite half-leaves of Nicotiana glutinosa L. were inoculated by the preformed RNP and TMV RNA, respectively.

b) Protoplast culture

Isolation of the mesophyll protoplasts from barley leaves, electroporation of the protoplasts with preformed MP-RNA complexes or viral RNA was carried out according to Zelenina et al. (1992) (FEBS Lett. 296 276-270). In a separate experiment the protoplasts were inoculated in the presence of polyethylene glycol (PEG) as described by Blum et al. (Virology 169, 51-61 (1989)).

Inoculated protoplasts were incubated for 24h at room temperature and TMV accumulation was determined by the ELISA double antibody sandwich method (DAS-ELISA) using serial TMV dilutions as concentration standards. Antisera to TMV were produced in rabbits by a series of intravenous and subcutaneous injections. Sheep anti-rabbit immunoglobulins were obtained from N.F. Hamaleya Institute of Epidemiology and Microbiology, Moscow.

RESULTS OF EXAMPLES 1, 2 AND 3

After incubation of ^{32}P-labelled crTMV RNA transcripts with the purified E.coli SSB protein and p50 preparations, the incubation mixtures were analyzed by a double filter nitrocellulose membrane filter binding assay described by Wong and Lohman (1993) (Wong, I., and Lohman, T.M. (1993), Proc. Natl. Acad. Sci. USA 90, 5428-5432).

This method is based on the principle that all RNP complexes are retained by the nitrocellulose, whereas protein-free RNA is trapped on the positively charged membrane placed beneath the nitrocellulose. Table 1 shows that at the E.coli SSB protein: RNA molar ratio of 100:1, 31% of the RNA was retained on the nitrocellulose membrane. In the same protein: RNA molar ratio p50 retains 60% RNA. However, at the ratio of 100:1 both E.coli SSB protein and p50 completely inhibit TMV RNA translation (Fig. 1 and 2 respectively).

In further experiments p50 was used. Table 2 and 3 show that p50:RNA complex preformed at the molar ratio 100:1 is noninfectious in protoplasts and in plants. These results imply that noninfectivity of the RNP complexes was due to their nontranslatability rather than to RNA degradation.

Table 1

Detection of the RNP complexes by nfb assays[a]

Preparation used	Molar protein: RNA ratio upon incubation	Per cent of radio-activity[b] retained on	
		Nitro-cellulose (protein-RNA complexes	Positively charged membrane (free RNA)
RNA		0.0	100.0
E.coli SSB protein - RNA complex	100:1	31.0±6.1	69.0±6.1
p50-RNA complex	100:1	60.1±4.6	39.9±4.6

[a] The mean values for 6 independent experiments are presented

[b] The total radioactivity of [^{32}P]-RNA retained on the nitrocellulose and on the positively charged membrane minus nonspecific background retention of free [^{32}P]-RNA on the nitrocellulose was taken as 100%. The nonspecific background retention was less than 1% of RNA radioactivity retained by a positively charged membrane.

Table 2

Examination of the ability of RNP complexes to infect protoplasts

Inoculum	Molar protein - RNA ratio upon preincubation	Amount of TMV accumulated in mesophyll protoplasts (ng per $5x10^6$) protoplasts[a]	
		Exp. 1	Exp.2
TMV RNA	-	650	1560
p50-RNA complex	100:1	0	0
Mock inoculation	-	0	0

[a] Protoplasts were electroporated with TMV RNA or TMV RNA preincubated with MPs. The amount of RMV RNA was 8 µg in 50 µl of inoculum. Concentration of TMV was determined by DAS-ELISA.

Table 3

Infectivity of TMV RNA complexes with the protein p50 of reticulocyte mRNPS

Protein	Infectivity: mean number of local lesions per half-leaf of N.glutinosa[a]			
	Experiment 1		Experiment 2	
	RNA	Protein/ RNA complex	RNA	Protein/ RNA complex
p50 - RNA complex	139±48.0	2±0.8	179±20.0	1±0.36

[a] Opposite halves of the same leaf of N.glutinosa were inoculated with the protein - TMV RNA complexes and free TMV RNA; the mean values for 8-10 inoculated leaves are presented in three independent experiments. The molar protein: RNA ratio was 100:1. 5 µl of inoculum applied to each half of the leaf contained 1 µg of TMV RNA.

EXAMPLE 4

Creation of rabbit p50 gene-containing transgenic tobacco

a) construction of rabbit p50 gene-containing Agrobacterium tumefaciens

The p50 gene was isolated by BamHI-EcoRV digestion of a p50 gene-containing plasmid Bluescript II SK+ (relevant part of the plasmid shown in Fig. 3A) and ligated into pRTαßGUS (Zelenina et al. (FEBS Lett. 296, 276-270 (1992), Fig. 3B) digested with XbaI (blunted with Klenow) and BamHI. This vector provides for the PVX nontranslated 5' leader sequence and a translational enhancer. Thus, the pRTαßp50 plasmid (Fig. 3C) was obtained.

The HindIII fragment from pRTαßp50 was inserted into pBin19 digested with HindIII to obtain pBinαßp50. A.tumefaciens was transformed with pBinαßp50 and seven positive clones were selected by Southern blot hybridization.

b) transformation of tobacco with p50 gene-containing A.tumefaciens LBA 4404

Nicotiana tabacum (Samsun) leaf disks were transformed with A.tumefaciens LBA 4404 containing pBinαßp50. Tobacco disks transformed with the transformed A.tumefaciens clones no. 1, 3, 5, 6 and 7 produced 60-70 shoots. 20 Shoots were taken for rooting and subsequent testing with rabbit polyclonal antibody against p50 produced in E.coli.

EXAMPLE 5

P50 gene under the control of a subgenomic viral promoter

In order to express P50, the gene is operably linked to the PVX coat protein subgenomic promoter. This chimeric construct is subsequently inserted in antisense orientation between the CaMV 35S promoter and terminator. Upon expression in transgenic plants activity of the CaMV 35S promoter results in RNA which contains a functional P50 gene which can be transcribed into a translatable mRNA only by the viral replicase. This construct is subsequently introduced in a transformation vector suitable for Agrobacterium-mediated plant transformation e.g. pBIN19 which contains the NPTII gene under control of the nopaline synthetase promoter for selection of transgenic shoots during the transformation procedure.

The binary vector is transferred to Agrobacterium tumefaciens LBA 4404 using a triparental mating procedure in which pRK2013 is used to mobilize the binary vector from Escherichia coli to Agrobacterium tumefaciens. The resulting Agrobacterium strain is used to transform Nicotiana tabacum L. Transformed tobacco plants are assessed for resistance by challenging with PVX.

CLAIMS

1. A method of producing a host organism which is resistant to infection by a virus comprising stably incorporating into the genome of said host a DNA sequence encoding a non-viral protein characterized in that said protein has the ability to bind to ssRNA from said virus to form a non-translatable RNA-protein complex.

2. The method according to claim 1 wherein said host organism is a plant.

3. The method according to claims 1 and 2, characterized in that the non-viral protein is selected from the group consisting of the SSB protein, the 56-60 kDa proteins which are responsible for global repression of mRNA in Xenopus oocytes and rabbit reticulocytes belonging to the Y box family of transcription factors and the reticulocyte 50kDa protein (p50).

4. A DNA sequence encoding a non-viral protein for use in the method as claimed in claims 1-3, wherein said protein has the ability to bind ssRNA from a virus to form a non-translatable RNA-protein complex.

5. DNA construct or vector harboring the DNA sequence as claimed in claim 4 in combination with suitable regulatory sequences (promoter, terminator, enhancer, nuclear localization signal etc.).

6. The DNA construct or vector as claimed in claim 5, wherein the promoter is a viral subgenomic promoter.

7. The DNA construct or vector as claimed in claim 6, wherein the viral subgenomic promoter is the promoter of the coat protein gene of Potato Virus X (PVX).

8. The DNA construct or vector as claimed in claims 5-7, further comprising a nuclear localization signal to ensure entry of the non-viral single stranded RNA binding protein into the nucleus.

9. The DNA construct or vector as claimed in claims 5-8 for use in transformation into a biological

system which allows expression of the encoded protein or an active part thereof.

10. Use of the DNA construct or vector as claimed in claims 5-8 in transformation into a biological system which allows expression of the encoded protein or an active part thereof.

11. Use as claimed in claim 10, wherein the suitable biological systems include yeast, cultured cells (such as insect cells, mammalian and plant cells) and plants and animals.

12. Host organism having improved resistance to viral infection said host containing recombinant DNA which expresses a non-viral protein capable of binding to ssRNA from said virus to form a non translatable RNA-protein complex.

13. Host organism as claimed in claim 12, wherein the host organism is a plant cell.

14. Plant cells transformed with recombinant DNA constructs or vectors according to claims 5-9.

15. Plant cells according to claim 13 or 14 for use in the regeneration of whole plants in which the new nuclear material is stably incorporated into the genome.

16. Transgenic plant having stably incorporated into its genome a DNA sequence encoding a non-viral protein having the ability to bind to ssRNA from said virus to form a non-translatable RNA-protein complex.

17. Transgenic plant as claimed in claim 16 obtainable by regenerating a plant cell according to claims 13 or 14 into a whole plant.

18. Transgenic plant as claimed in claim 16 or 17 for use as a parent in standard plant breeding crosses to develop hybrids and lines having improved viral resistance.

19. Seeds showing improved resistance to viral infection and having stably incorporated into their genome a recombinant DNA sequence encoding a non-viral protein having the ability to bind to ssRNA from said virus to form a non-translatable RNA-protein complex.

20. Seeds as claimed in claim 19 obtainable from transgenic plants as claimed in claims 16-18 or their progeny.

21. Progeny of transgenic plants as claimed in claims 16-18 showing improved resistance to viral infection and having stably incorporated into their genome a recombinant DNA sequence encoding a non-viral protein having the ability to bind to ssRNA from said virus to form a non-translatable RNA-protein complex.

22. Progeny as claimed in claim 21 obtainable by germinating seeds as claimed in claim 19 or 20.

23. Genetically modified plant cells, plants or seeds as defined in claims 13-21 belonging to field crops, cereals, fruit and vegetables such as canola, sunflower, tobacco, sugarbeet, cotton, soya, maize, wheat, barley, rice, sorghum, tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, melons, potatoes, carrot, lettuce, cabbage, onion.

24. Recombinant protein having the ability to bind ssRNA from a virus to form a non-translatable RNA-protein complex for use in providing virus resistance to an organism, in particular a plant or animal.

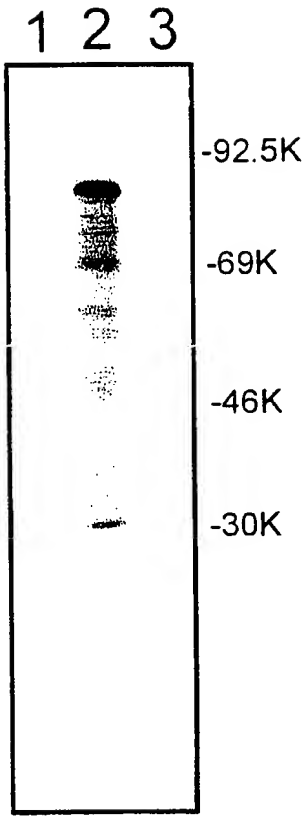


Figure 1

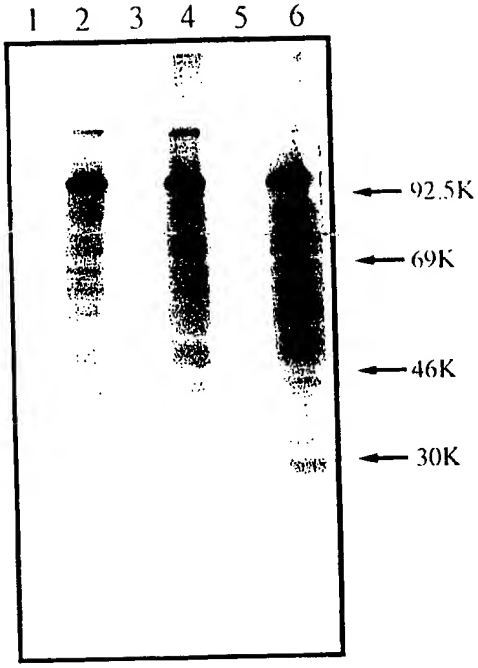
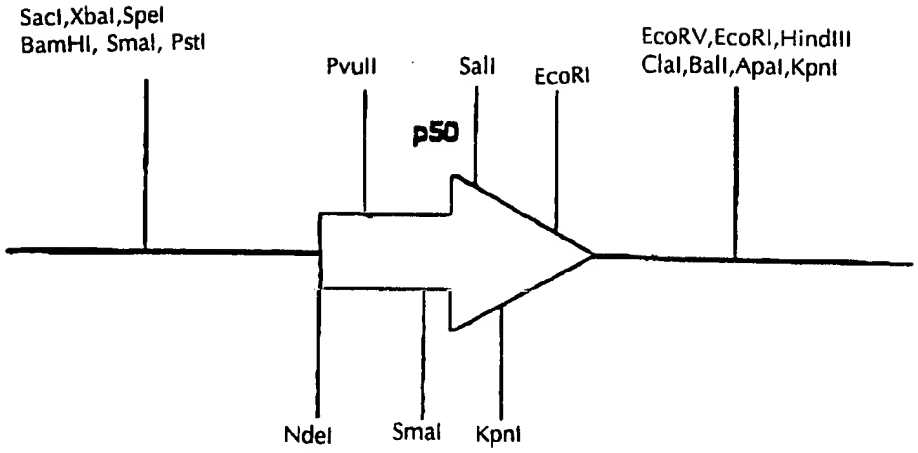
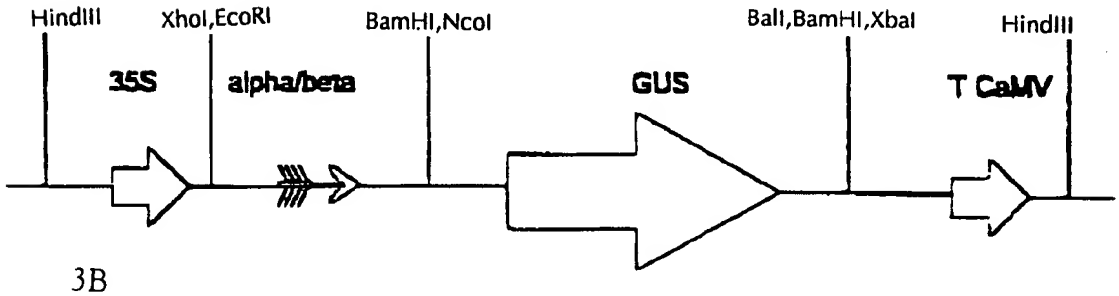


Figure 2

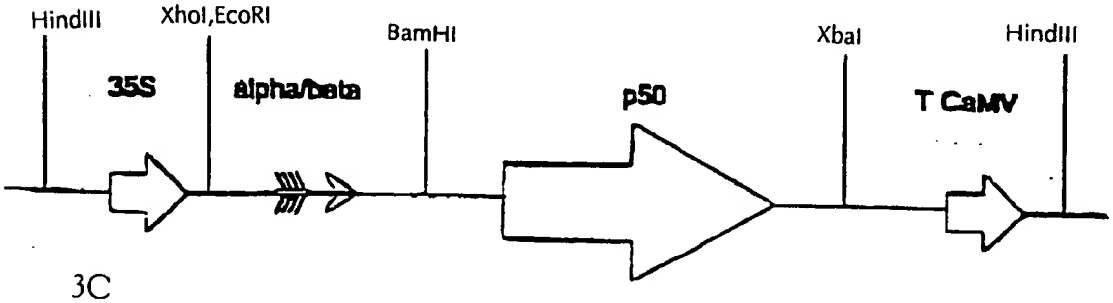
FIGURE 3



3A



3B



3C

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/EP 98/01925

A. CLASSIFICATION F SUBJECT MATTER
IPC 6 C12N15/82 C07K14/46 C07K14/47 C07K14/245 C12Q1/68
G01N33/53

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K C12Q G01N

Documentation searched other than minimumdocumentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BECK, D.L., ET AL. : "disruption of virus movement confers broad-spectrum resistance against systemic infection by plant viruses with a triple gene block" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 91, October 1994, pages 10310-10314, XP002073439 abstract, page 10310; 10311, right column, page 10313, 10314, Fig. 3 ---	1,2,4,5, 9-18, 20-24
Y	KALININA, N.O., ET AL.: "expression and biochemical analyses of the recombinant potato X 25K movement protein" FEBS LETTERS, vol. 397, 1996, pages 75-78, XP002073440 cited in the application page 77, right column, last paragraph --- -/--	1,2,4,5, 9-18, 20-24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of theinternational search	Date of mailing of the international search report
3 August 1998	20/08/1998

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INTERNATIONAL SEARCH REPORT

Inte. onal Application No
PCT/EP 98/01925

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	LAPIDOT, M., ET AL. : "a dysfunctional movement protein of tobacco mosaic virus that partially modiies the plasmodesmata and limits virus spread in transgenic plants" THE PLANT CELL, vol. 4, no. 6, 1993, pages 959-970, XP002073441 see also page 960, left column see the whole document ---	1,2,4,5, 9-18, 20-24
Y	IVANOV, K.I., ET AL. : "the immobilized movement proteins of two tobamoviruses form stable ribonucleoprotein complexes with full-length viral genomic RNA" FEBS LETTERS, vol. 346, 1994, pages 217-220, XP002073442 cited in the application see the whole document ---	1,2,4,5, 9-18, 20-24
A	MITRA, A., ET AL. : "a mammalian 2-5A system functions as an antiviral pathway in transgenic plants" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 93, June 1996, pages 6780-6785, XP002073443 page 6780 , left column ---	1-24
A	CITOVSKY, V., ET AL.: "the p30 movement protein of tobacco mosaic virus is a single-strand nucleic acid binding protein" CELL, vol. 60, February 1990, pages 637-647, XP002073444 cited in the application abstract, page 639,640,641,64,645; Fig. 2,3,9 ---	1-24
A	WO 94 00012 A (GENE SHEARS PTY LTD ;ATKINS DAVID G (AU); GERLACH WAYNE L (AU); YO) 6 January 1994 pages 10,11,13 ---	1-24
A	WO 96 40948 A (RES CORP TECHNOLOGIES INC) 19 December 1996 abstract, pages 7,19,20; page 17, line 15; examples 1,5,6,7, claims --- -/--	1-24

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INTERNATIONAL SEARCH REPORT

Inte. .onal Application No
PCT/EP 98/01925

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	EVDOKIMOVA, V.M., ET AL. : "the major protein of messenger ribonucleoprotein particles in somatic cells is a member of the Y-box binding transcription factor family" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 7, 1995, pages 3186-3192, XP002073445 cited in the application see the whole document ---	1-24
A	ATABEKOV J G: "NEW STRATEGIES FOR CONSTRUCTION OF VIRUS RESISTANT TRANSGENIC PLANTS" MITTEILUNGEN AUS DER BIOLOGISCHEN BUNDESANSTALT FUER LAND UND FORSTWIRTSCHAFT, vol. 309, 10 April 1995, page 23/24 XP002047297 see the whole document ---	7
P,Y	SEPPÄNEN,P., ET AL. : "movement protein-derived resistance to triple gene block-containing plant viruses" JOURNAL OF GENERAL VIROLOGY, vol. 78, June 1997, pages 1241-1246, XP002073446 see the whole document -----	1,2,4,5, 9-18, 20-24

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ional Application No
PCT/EP 98/01925

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9400012 A	06-01-1994	AU 683619 B	20-11-1997
		AU 4545093 A	24-01-1994
		CA 2137161 A	06-01-1994
		EP 0652705 A	17-05-1995
		FI 946121 A	28-12-1994
		HU 71929 A	28-02-1996
		JP 8500971 T	06-02-1996
		NO 945018 A	23-12-1994
		NZ 253963 A	22-08-1997
WO 9640948 A	19-12-1996	EP 0832260 A	01-04-1998